Identification of Substrate Binding Domains in Human Nucleobase Transporters

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Proposed Plan of Study

Introduction

Cells must employ special carrier proteins to move solute molecules across the lipid bilayer. This uptake is especially critical for any cell types lacking *de novo* synthesis of important molecules. Nucleoside and nucleobase transportation is common in a large variety of organisms and has many different physiological effects (Griffith and Jarvis 1996). Cytotoxic nucleosides are used in treatment of human neoplastic and viral diseases (Clumeck 1993); human immunodeficiency viral (HIV) infections are commonly treated with the nucleoside analog azidothymidine (AZT). Cancer and cardiac arrhythmias are also treated by other therapeutic nucleoside analogs (Lerman and Belardinelli 1991, Mani and Ratain 1996). Functional characterization of these transporters is, therefore, important for understanding uptake of nucleoside drugs (Cass 1995) in their use for treating human disease.

Nucleoside transporters are divided into two functional categories: equilibrative and concentrative. Equilibrative transporters are passive and exhibit broad substrate specificity for purine and pyrimidine nucleosides. Many cell types express equilibrative types and are common in mammals. Concentrative nucleoside transporters are classified by their substrate selectivity for purines, pyrimidines, or both. They actively increase intracellular concentrations by coupling to the Na^+ gradient and they are thought to exist only in specialized cells.

Though not as well known, nucleobase transporters are also found in mammalian cells. Additionally, many non-mammalian eukaryotes employ transporters for uptake of nucleobases. *Saccharomyces cerevisiae* has a purine-cytosine specific transporter encoded by the FCY2 gene (Weber *et al.* 1990) and a uracil-specific transporter encoded by FUR4 (Jund *et al.* 1988).

Human nucleoside transporter clones exist for both equilibrative (Griffiths *et al.* 1997a, Griffiths *et al.* 1997b), and concentrative (Wang *et al.* 1997) types, but human nucleobase transporter genes remain undiscovered. Kinetic uptake studies show nucleobase transporters exist in mammalian cells, but their numbers and specificities are unclear (Griffith and Jarvis 1996). In human erythrocytes, both hypoxanthine and adenine are thought transported by the same transporter (Plagemann *et al.* 1987, Kraupp *et al.* 1991). We know very little, however, about the molecular mechanisms and domain functions in either nucleobase or nucleoside transporters.

Here I propose to find and characterize the substrate binding domains of uracil and purine-cytosine human nucleobase transporters by taking advantage o homologies with the yeast transporter genes FCY2 and FUR4. Describing these transporters will lead to new insights into the uptake and molecular design of nucleobase analog drugs for human disease therapy.

Project Specific Aims

The primary goal of this project is to identify and characterize the protein domains responsible for substrate specificity in one or more human nucleobase transporters. Achieving this will require successful completion of three main objectives.

1. Find and clone human genes homologous to the *S. cerevisiae* genes FCY2 and FUR4.
2. Characterize uptake and inhibition of human nucleobase transporters in *Xenopus* oocytes.
3. Determine human nucleobase transporter protein domain functionality.

Research Methods

1. **Find and clone human genes homologous to the FCY2 and FUR4 genes in yeast.** Clones of these genes are already well characterized in *S. cerevisiae* (Weber *et al.* 1990, Jund *et al.* 1988). I plan to use two different methodologies to screen for their existence in a human glial blastoma cDNA library in a yeast vector. First, I will use the FCY2 and FUR4 published sequences to design polymerase chain reaction (PCR) primers for amplification of any homologous regions in the human cDNA library. The primers will be designed to bind to regions likely conserved between yeast and humans and will be degenerate to compensate for possible synonymous differences. I will sequence any PCR products close to the target length, then design upstream and downstream primers to further sequence any human PCR products showing homology with the yeast genes. The second method involves using a complementation test to assay for human cDNA clones able to restore transport in knockout yeast. I will transform FCY2 and FUR4 knockout strains of *S. cerevisiae* with a yeast expression vector containing a human cDNA library. Any human clones able to restore substrate uptake will then be sequenced.
2. **Characterize uptake and inhibition of human nucleobase transporters in *Xenopus* oocytes.** To test the uptake characteristics of the cloned nucleobase transporters identified by PCR or complementation, I will produce mRNA by using *in vitro* reverse transcription. The messenger RNA will then be microinjected directly into *Xenopus* oocytes and measured for their uptake activity using radioactive substrates (Chandrasema *et al.* 1997). I will also test uptake inhibition with known nucleobase transporter inhibitors such as papaverine (Kraupp 1995).
3. **Determine human nucleobase transporter protein domain functionality.** Identifying roles of particular regions of the transporter proteins will be necessary to localize sites of substrate specificity; therefore, I will produce both point mutation and chimeric transporters. I will first use *in vitro* chemical mutagenesis to make random mutations in vectors containing the cloned transporter gene and assay for loss of substrate uptake. Any substrate-specific region identified by mutation analysis will then be exchanged with a homologous domain from another transporter type as Wang and Giacomini (1997) did with nucleoside transporters. I will use *in vivo* 'sticky-feet'-directed mutagenesis (Clackson and Winter 1989) to make the chimeric transporters.

Progress to Date

During the last six months I have searched for human nucleobase transporters using both PCR and complementation. The primers I designed to amplify a 450bp region of FUR4 in *S. cerevisiae* have produced several products from a human glial blastoma cDNA library. One of these products is ~550bp long; its sequencing is not yet complete. I have also obtained strains *S. cerevisiae* strains containing knockout versions of FCY2 and FUR4. My transformations of the FUR4 knockout strain with the human cDNA library have yet to produce a clone able to restore uracil transportation. To identify domains likely mediating substrate binding, I did a phylogenetic analysis of all published nucleobase and nucleoside transporter protein sequences. I found transmembrane domains in all of these transporters are evolving about 1.5 times slower than non-transmembrane domains. Their conservation suggests transmembrane domains may confer substrate specificity and are good targets for mutagenesis.

Feasibility

I will spend the first year searching for FCY2 and FUR4 homologs in the glial blastoma human cDNA library using both PCR and complementation. If they remain unfound, I will obtain another human cDNA library or produce my own. The second year I will characterize the clones using kinetic studies of transporter activity in *Xenopus* oocytes. By the third year I will be identifying the substrate-specific domains using point and chimeric mutations.

This project's highest risk is in finding human homologs of FCY2 and FUR4. If they are not found, I will switch to identifying substrate-specific domains in the equilibrative nucleoside transporters already cloned and characterized. Because my phylogenetic analysis shows little overall similarity between FCY2 and FUR4, there is also some risk in finding suitable homologous regions in two nucleobase transporters for the chimeric domain swaps. Specific regions, however, are homologous, particularly in the conserved transmembrane regions.

Literature Cited

Cass CE. (1995) in Drug Transport in Antimicrobial and Anticancer Chemotherapy (Georgopapakou, N.H., ed). pp. 403-451, Marcel Dekker, New York.

Chandrasema G, Giltay R, Patil SD, Bakken A, and Unadkat JD. (1997) Functional expression of human intestinal Na^+-dependent and Na^+-independent nucleoside transporters in *Xenopus laevis* oocytes. *Biochem. Pharmacology* 53:1909-1918.

Clackson T, and Winter G. (1989) 'Sticky-feet'-directed mutagenesis and its application to swapping antibody domains. *Nuc. Acids. Res.* 17:10163-10170.

Clumeck N. (1993) Current use of anti-HIV drugs in AIDS. *J. Antimicrobial. Chemother*. 32: Suppl. A, 133-138.

Griffith DA, and Jarvis SM. (1996) Nucleoside and nucleobase transport systems of mammalian cells. *Biochim. Biophys. Acta* 1286:153-181.

Griffiths M, Beaumont N, Yao SYM, Sundaram M, Boumah CE, Davies A, Kwong FYP, Coe I, Cass CE, Young JD, and Baldwin SA. (1997a) Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. *Nature Medicine* 3(1):89-93.

Griffiths M, Yao SYM, Abidi F, Phillips SEV, Cass CE, and Young JD. (1997b) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (*ei*) equilibrative transporter from human placenta. *Biochem. J.* 328:739-743.

Jund R, Weber E, and Chevallier MR. (1988) Primary structure of the uracil transport protein of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 171(1-2):417-424.

Kraupp M, Marz R, Prager G, Kommer W, Razavi M, Baghestanian M, Chiba P. (1991) *Biochim. Biophys. Acta* 1070:157-162.

Kraupp M. (1995) Membrane transport of nucleobases: interaction with inhibitors. *Gen. Pharmacol.* 26:(6)1185-1190.

Lerman BB, and Belardinelli L. (1991) Cardiac electrophysiology of adenosine. Basic and clinical concepts. *Circulation* 83:1499-1509.

Mani S, and Ratain MJ. (1996) Promising new agents in oncologic treatment. *Curr. Opin. Oncol.* 8:525-534.

Plagemann PGW, Woffendin C, Puziss M, and Wohlhueter RM. (1987) Purine and pyrimidine transport and permeation in human erythrocytes. *Biochim. Biophys. Acta* 905:17-29.

Wang J, and Giacomini KM. (1997) Molecular determinants of substrate selectivity in Na+-dependent nucleoside transporters. *J. Biol. Chem.* 272(46): 28845-28848.

Wang J, Su SF, Dresser MJ, Schaner ME, Washington CB, Giacomini KM. (1997) Na^+-dependent purine nucleoside transporter from human kidney cloning and functional characterization. *Am. J. Physiol.* 273 (*Renal Physiol.* 42):F1058-F1065.

Weber E, Rodriguez C, Chevallier MR, and Jund R. (1990) The purine-cytosine permease gene of *Saccharomyces cerevisiae*: primary structure and deduced protein sequence of the FCY2 gene product. *Mol. Microbiol*. 4(4):585-596.